Chemical perturbation of Mcl-1 pre-mRNA splicing to induce apoptosis in cancer cells

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Supporting Information

METHODS

Compound treatment. ABT-737 was purchased from Selleck Chemicals. Meayamycin B was synthesized in our laboratory. The compounds were dissolved in dimethyl sulfoxide (DMSO) to make 10 mM stock solutions and stored at –20 °C. For 96-well format assays, we diluted the compounds with cell culture medium and transferred each dilution (100 μL) to cell cultures (100 μL) in triplicate. For 35-mm dish-format assays, cells were seeded in 35-mm dishes and cultured for 24 h (~70% confluence) before vehicle or test compounds were added directly from stock at appropriate concentrations. In both cases, the final vehicle% was controlled at 0.5% for all of the treatments. After incubation for the indicated periods of time (1, 3, 9, and 24 h for time-dependence experiments, 9 or 72 h for dose-dependence experiments), the cells in 96-well plates were lysed and subjected to luciferase assays or caspase-3/7 assays, and the cells from 35-mm dishes were subjected to total RNA extraction and the RT-PCR experiments.

Apoptosis detection: (1) FITC-, annexin V-, and 7-aminoactinomycin D (7-AAD) staining — Cells (1×10^6 cells) were treated for 9 h with 10 nM meayamycin B, 5 μM ABT-737, a combination of these compounds, or an equal volume of DMSO as a negative control. After treatment, the cells were harvested, washed in ice-cold PBS, and directly stained with 5 μg mL^{-1} FITC Annexin V (BD Pharmingen, cat. no. 556420) and 2.5 μg mL^{-1} 7-AAD (BD Pharmingen, cat. no. 559925). After 20 min of incubation at room temperature in the dark, cells were analyzed by flow cytometry using a Beckman Coulter Epics XL-MCL. Data were analyzed using Summit V4.3 software.

(2) Caspase 3/7 activity assays — The cells were seeded at 1×10^4 per well in medium (100 μL) in white solid-bottom 96-well plates and cultured for 24 h. Meayamycin B (0.1, 1, 10, and 100 nM) and ABT-737 (0.05, 0.5, 5, and 50 μM), either separately or in combination (constant ratio of 1:500), were added in duplicate into the cells for 9 h. Caspase-3/7 activity was quantified using a Caspase-Glo® 3/7 reagent (Promega, cat. no. G8091) following the manufacturer's optimized protocol. Specifically, the Caspase-Glo® 3/7 buffer and the substrate were equilibrated to room temperature and mixed immediately before assays. This conjugated assay buffer (100 μL) was added to a cell culture (100 μL) that was equilibrated to room temperature, and the mixture was incubated at room temperature for 1 h. Luminescence was directly measured with a Modulus II Microplate

Multimode Reader. The caspase-3/7 activity was expressed as the mean luminescence of compound-treated wells divided by that of vehicle-treated wells.

Semi-quantitative reverse transcription-Polymerase Chain Reaction (RT-PCR). The total RNA was extracted using a Trizol reagent (Invitrogen, cat. no. 15596-026) and cDNA generated by reverse transcription using 1 μg of total RNA and SuperScript® II reverse transcriptase (Invitrogen, cat. no. 18064-014). The primer sequences are shown in Table S1. For semi-quantitative RT-PCR, the thermocycler program for Bcl-x, Mcl-1 and β-actin involved an initial denaturation at 94 °C for 5 min, 35 cycles at 94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 50 sec, and a final elongation at 72 °C for 7 min. The PCR products were examined on 1.5% agarose gels containing 0.5 μg mL^{-1} ethidium bromide and imaged by a Molecular Imager Gel Doc™ XR+ (BioRad). The intensity of the bands was quantified with the Lab Imager software (BioRad).

Immunoblotting. Cells were harvested in a cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride) and a mixture of protease inhibitors (Roche, cat. no. 05 892 791 001). Total protein (100 μg) was electrophoresed on a 12% SDS-polyacrylamide gel and electrotransferred to a polyvinylidene difluoride membrane (Millipore, cat. no. IPVH10100). Membranes were blocked at room temperature for 1 h with a blocking buffer (5% non-fat dry milk in 10 mM Tris-HCl pH7.6, 150 mM NaCl, 0.1% Tween-20) and then incubated at 4 °C overnight with rabbit anti-Mcl-1 monoclonal antibody (1:1000 dilution; Cell signaling technology, cat. no. 5453S), rabbit anti-Bcl-$x_{L/S}$ monoclonal antibody (1:1000 dilution; Santa Cruz, cat. no. sc-634) or rabbit anti-β-actin monoclonal antibody (1:1000 dilution; Cell signaling technology, cat. no. 4970S), followed by 1 h of incubation with horseradish peroxidase-conjugated anti-rabbit (1:1000 dilution; Cell signaling technology, cat. no. 7074S) secondary antibody. Blots were developed with ECL Plus reagents (PerkinElmer Life and Analytical Science, cat. no. NEL103001EA). Mcl-1_L, Mcl-1_S, Bcl-x_L, Bcl-x_S, and β-actin proteins migrated at 40, 35, 30, 26, and 45 kDa, respectively. The densities of the resulting bands were quantified using Image Gauge Ver. 4.0 (FUJIFILM).

Statistics. Data analysis and graph plotting were carried out using a GraphPad Prism 5.0c for Mac (GraphPad Software). All the data were presented as mean ± standard deviation. The significance level for all analyses was 5%.

Table S1. Primer sequences for RT-PCR experiments

	Sequences
Bcl-x	Bcl-xF: 5′- GAG GCA GGC GAC GAG TTT GAA -3′ Bcl-xR: 5′- TGG GAG TTG AGA GTG GAT GGT -3′
Mcl-1	Mcl-1F: 5′- ATC TCT CGG TAC CTT CGG GAG C -3′ Mcl-1R: 5′- CCT GAT GCC ACC TTC TAG GTC C -3′
	Mcl-2F: 5′- AGG AAT TCG ATG TTT GGC CTC AAA AGA AAC GCG GTA -3′ Mcl-2R: 5′- GAA TTC GGA AGT TAC AGC TTG GAG GAG TCC AAC TGC -3′
β-actin	Forward: 5′- GCA CCA CAC CTT CTA CAT GAG C -3′ Reverse: 5′- TAG CAC AGC CTG GAT AGC AAC G -3′

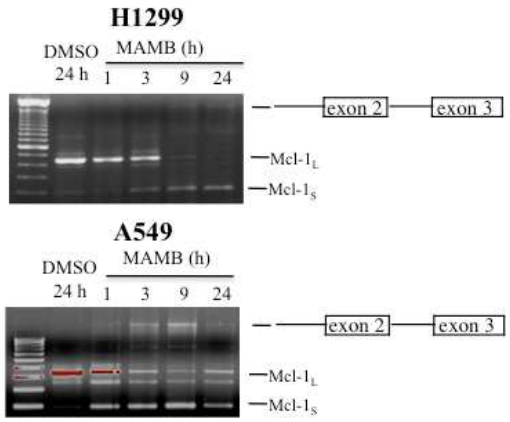

Figure S1. Meayaymycin B (MAMB) inhibits the constitutive splicing of *MCL1* gene in H1299 and A549. Cells were exposed to 10 nM MAMB for various durations before relative levels of Mcl-1 splicing variants were assessed using semi-quantitative RT-PCR. Cells exposed to equal volume of DMSO were used as negative controls. Data represent results from three separate experiments.